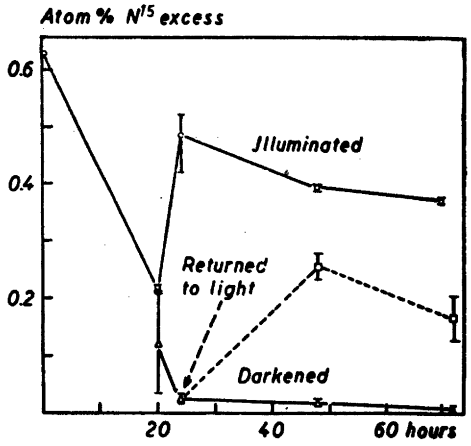

Fig. 1. Influence of illumination of pea plants on fixation by their nodules when exised. Vertical lines define the extreme values of replicate samples.

and exhibited a marked decrease in leghaemoglobin. The nodules from the illuminated plants were indistinguishable from those of the darkened plants which had been returned to light.

It seems most likely that the rapid decay of the nitrogen fixing capacity of nodules from plants kept in the dark for 24 h for the most part arises from depletion (and their rapid recovery in the light from renewal) of substrates furnished normally by the photosynthesizing plant, but partly also from the decrease in leghaemoglobin in the nodules. Virtanen [2] has shown that the oxydation of this pigment to a green one checks N_2-fixation, and a recent investigation by Virtanen and Berg has shown that a smaller part of leghaemoglobin is destroyed even during 24 h in darkness. Thus it can be understood that the transfer of plants to light causes a partial recovery of the effectivity of the root nodules, but far from the whole of it.

Whatever the basis for the observed response may be, the data clearly show that the nodules from pea plants kept in the dark have far less capacity for fixing N_2 than nodules from illuminated plants, and that the lost activity can be partially recovered by returning the plants to the light. It would be of interest to employ the isotopic method to determine in greater detail the rate of decay and recovery of nitrogen fixing capacity, the time required before the injury to the nitrogen fixing capacity becomes irreversible, and the influence of light intensity on the fixation process.

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Estimation of Keto Acids in Plants

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In the study of keto acids the paper chromatography of their 2,4-dinitrophenylhydrazones has been used. As the hydrazones of keto acids form multiple spots on the chromatogram, thus making the identification of the spots uncertain, unknown keto acids are difficult to discover. In order to elucidate unknown acid hydrazones in urine, Kulonen [1] reduced the hydrazones to the corresponding amino acids with aluminium amalgam according to the old method reported by Fischer [2], and investigated the amino acids by paper chromatography. Later Towers, Thompson, and Steward [3] used a similar method, the reduction being made catalytically. We have also developed a modification of the procedure which has already given some interesting results regarding keto acids in plants [4,5]. An account of it is given in the present paper. In our method the reduction is achieved by tin in an alcoholic hydrochloric acid solution, the yield of hydrogenolysis being comparatively good. The purification of the acid hydrazones obtained from the plant material was made according to Virtanen *et al.*[6]

Procedure. 1. *Preparation of the plant extract.* The plant material (usually about 100 g fresh wt.) was homogenized 3 times in a

tungstic acid solution prepared just before use by mixing water, 10 % sodium tungstate, and 2/3 *N* sulphuric acid in the proportion 60:20:20 (v/v) according to Cavallini and Frontali [7]. The homogenate was filtered through a cheese cloth, the filtrates were combined and refiltered, this time through filter paper. The proportion between plant material and total extracting solution was in most cases 1:5 (w/v).

2. *Reaction with dinitrophenyl hydrazin.* The combined extracts were mixed with a 1 % solution of 2,4-dinitrophenyl hydrazine in 5 *N* H_2SO_4 (1 ml/g fresh wt. of plant material, Virtanen *et al.*[6]), and the reaction mixture was allowed to stand at room temperature for about 1 h. If a heavy precipitate was formed during the reaction the mixture was filtered once more.

3. *Extraction.* The reaction mixture was extracted according to Virtanen *et al.*[6] with ethyl acetate, and the ethyl acetate solution was extracted with 10 % Na_2CO_3. The combined soda phases were then acidified to about pH = 1 with 3 *N* H_2SO_4 and extracted with ethyl acetate. By this treatment the keto acid hydrazones could be separated from most of the amino acids and neutral carbonyl compounds in the original plant extract.

4. *Hydrogenolysis.* The final ethyl acetate extract was evaporated *in vacuo*, the residue was dissolved in 95 % ethanol and an aliquot (usually 2/3 or 4/5 of the solution, 1/3—1/5 being used as a control; see steps 6 and 7) was hydrogenated with tin by passing gaseous hydrochloric acid through the solution until a strong evolution of hydrogen gas started. Previously prepared HCl-ethanol was used in some cases. The vessel used in the hydrogenation was kept in an ice-water bath. The reaction time used varied from 12—20 h.

5. *Precipitation of the tin.* The reaction mixture was filtered, diluted with water, and the tin-ions were precipitated with hydrogen sulphide. The precipitate was then filtered off and the filtrate taken to dryness *in vacuo*.

6. *Purification with ion-exchange.* The residue was dissolved in water and passed through an Amberlite IR-120 column (25 × 1.3 cm). After washing the column with water the amino acids formed during the hydrogenolysis were displaced with 1 *N* NH_3. The amino acid solution which often had a brown colour was concentrated *in vacuo* or on a waterbath. The colour did not seriously interfere with the subsequent paper chromatography. The remaining non-hydrogenated keto acid hydrazones from step 4 were treated with Amberlite IR-120 in a similar way.

7. *Paper chromatography.* The amino acids formed were identified by two-dimensional chromatography using Whatman No. 4 paper and butanol-acetic acid and phenol + NH_3 as solvents. A corresponding amount of the similarly treated non-hydrogenated keto acid hydrazones was chromatographed as a control. It never contained more than traces of some amino acids present as contaminants.

Quantitative considerations. As to the quantitativity of the method we have obtained the following yields when treating known amounts of keto acids in accordance with the directions above: pyruvic acid 78 %, α-keto-butyric acid (impure prep.) 32 %, oxalacetic acid 61 %, and α-ketoglutaric acid 43 %. The values are approximate obtained by comparing the spots of the amino acids formed with spots of the corresponding amino acids in different concentrations. By starting directly from the synthetic hydrazones the yield for α-keto-butyric acid rises to about 60 %. Towers, Thompson, and Steward [3], using catalytical hydrogenation, got a yield of 65.3 % with pyruvic acid hydrazone, 97 % with glyoxylic acid hydrazone, but only 33.0 % and 36 % with the hydrazones of oxalacetic and α-ketoglutaric acid, respectively. Kulonen [1] in turn reports that the method of Fischer which he used for reduction is very unsatisfactory for the investigation of α-ketoglutaric acid, or of other dicarboxylic keto acids. The method for reduction used by us has, as mentioned above, given relatively good results both with ketodicarboxylic acids and keto-monocarboxylic acids. This has made the discovery of several new ketodicarboxylic acids possible [4,5]. The above mentioned values show that quantitative considerations are possible only if the yield of the hydrazone hydrogenolysis is known for each keto acid in question, the actual amounts may thus be 30—70 % higher than the amounts found on the chromatograms, in some cases possibly even higher. Our experiments in agreement with those of Towers, Thompson, and Steward [3] have also shown that 2,4-dinitrophenylhydrazone + ascorbic acid or dehydroascorbic acid does not give rise to noticeable amounts of amino acids during the hydrogenation procedure. The ninhydrin spots on chromatograms made from plant extracts by this method accordingly do not contain compounds derived from the above-mentioned substances.